The 30th Edward F. Hayes Graduate Research Forum

Cláudio M. Vrisman

Dynamics of Colonization of Cucurbits by the Bacterial Plant Pathogen *Erwinia tracheiphila*

This paper is presented as a requirement for winning first place at The 30th Edward F. Hayes Graduate Research Forum in Columbus, Ohio on February 26, 2016. The presentation was performed at the College of Food, Agricultural, and Environmental Sciences (CFAES) section.

Detailed information about the work presented can be found in the following publication: Vrisman, C. M., Deblais, L., Rajashekara, G., Miller, S. A. 2016. Differential colonization dynamics of cucurbit hosts by *Erwinia tracheiphila*. Phytopathology (http://dx.doi.org/10.1094/PHYTO-11-15-0289-R).

Cucurbits are important crops in the agricultural economy with more than 110,000 hectares of cucumber, melon, pumpkin, and squash cultivated annually for fresh and processing markets in the United States (NASS 2015). Cucumbers provide a good source of vitamins A, K, and C, as well as large amounts of potassium. Pumpkins are an excellent source of carotenoids and vitamins A, B6, C, and E, as well as iron, magnesium, phosphorous, potassium, copper, and manganese.

Many plant pathogens can affect cucurbit crops and destroy entire fields. Bacterial wilt of cucurbits, caused by *Erwinia tracheiphila* (E. F. Smith) Holland, is considered one of the most destructive diseases of cucurbits grown in the Midwestern and Northeastern U.S., and losses can go as high as 80% (Rojas et al. 2015). This disease is present in the United States, Europe, South

Africa, and Japan (Agrios 2005). Among all the genera in the Cucurbitaceae family, two have been the focus of research with this pathosystem: 1) *Cucumis*, the genus of melon and cucumber, the center of origin of which is in Africa and India; and 2) *Cucurbita*, the genus of pumpkin and squash, the center of origin of which is in the Mesoamerica (Sebastian et al. 2010). Watermelon (*Citrullus* genus) was an unknown host until recent report of its occurrence in New Mexico (Sanogo, Etarock, and Clary 2011). Management of this disease has relied on the use of insecticides and row covers, among other approaches described in depth in Rojas et al. (2015).

Host colonization dynamics remain unclear for this understudied pathogen. The pathogen is introduced via feeding wounds caused by its insect vectors, both striped (*Acalymma vittatum* (F)) and spotted (*Diabrotica undecimpunctata howardi* Barber) cucumber beetles. These beetles feed on all cucurbit plant parts through the growing season. The pathogen is transmitted via the vector's feces deposited in or near beetle feeding wounds (Brust 1997a, 1997b; Yao et al. 1996). Younger plants are more susceptible than older ones and can wilt within 6 days after infection (Liu et al. 2013). Wilting occurs due to the multiplication of bacteria inside xylem tissue, blocking water movement (Koike, Gladders, and Paulus 2007). *Erwinia tracheiphila* colonization alters foliar volatile emissions. Cucumber beetles are attracted to volatiles from wilting foliage; however, the vectors are more attracted to volatiles of flowers of healthy plants, indicating an efficient way for dispersal of this pathogen from plant to plant (Shapiro et al. 2012). Understanding the plant-pathogen interaction at different crop stages, and how the decrease in plant susceptibility occurs as plants age can have useful implications for disease management.

Erwinia tracheiphila has been studied for more than a century. Preference of an *E. tracheiphila* strain isolated from muskmelon to infect cucumber over squash was reported as early as 1905 (Smith 1911). However, only recent reports showed genetic variability among *E.*

tracheiphila strains associated with a preference for colonizing hosts in the genus from which they were isolated. Genetic variability was reported among 69 strains isolated from five different species, with a distinct pattern between *Cucumis* and *Cucurbita* genera. Summer squash seedlings inoculated with *Cucurbita*-derived strains wilted 6 days after inoculation, while those inoculated with *Cucumis*-derived strains showed few or no wilting symptoms. Symptomatic plants wilted 18 days after inoculation. When muskmelon seedlings were inoculated with a *Cucurbita*-derived strain, symptoms developed approximately 11 days later than seedlings inoculated with a *Cucumis*-derived strain (Rojas et al. 2013). A similar study performed by Nazareno and Dumenyo (2015) showed similar results for cross-inoculation studies. The differences between productive and non-productive plant colonization are not yet very well understood. A bioluminescent strain of a *Cucumis*-derived *E. tracheiphila* was constructed to describe colonization dynamics in melon, cucumber, squash, and pumpkin plants (Vrisman et al. 2016). The study by Vrisman et al. (2016) was the first to describe, visualize, and quantify the colonization dynamics of *E. tracheiphila* inside preferred and non-preferred cucurbit hosts.

Bioluminescence imaging permits real-time and non-destructive monitoring of bacterial colonization, thus providing reliable analysis of pathogen location while reducing host variability. The technique relies on constructing bacterial strains harboring the *lux* operon. Transformed cells emit photons that can be captured by a sensitive charge coupled device (CCD) camera allowing visualization of bacterial cells within plant tissue. Natural light emission is encoded by the *luxCDABE* genes, where *luxA* and *luxB* are responsible for coding α and β subunits, respectively, of the catalytic enzyme. The three other genes (l*uxC, luxD,* and *luxE*) encode the subunits of a fatty acid reductase (Meighen 1993; Stewart et al. 1992; Xu et al. 2010). The bioluminescent system is

best studied in *Photorhabdus luminescens*, which is known to possess the *lux* operon (Kassem et al. 2014).

Bacteria transformed with bioluminescent genes were first used to study pathogenesis in animals. The use in plant pathogens is recent and it has been reported for other pathosystems within the *Erwinia* genus, for example, where bioluminescent *E. amylovora* was used to study colonization of apple plants (Bogs et al. 1998, Azegami et al. 2004). Bioluminescent bacteria were also used to study the colonization dynamics of *E. ananas* in rice plants (Hasegawa et al. 2003). Bioluminescence was also used to describe colonization of potato plants by the destructive pathogen *Ralstonia solanacearum*. Latent infections were detected, indicating that colonization of potato plants occurs well before symptom expression (Cruz et al. 2013). Colonization of tomato plants by Gram-positive bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* was first demonstrated by Xu et al. (2011), who described upward and downward movement of this pathogen and differential response to relative humidity in growing environments.

Vrisman et al. (2016) developed a bioluminescent strain of *E. tracheiphila* that was used to study its colonization via different routes of inoculation and to understand the colonization dynamics of a *Cucumis*-strain when inoculated on different cucurbit hosts. The wild type strain used, TedCu10, was isolated from *Cucumis sativus* (cucumber). Cells of TedCu10 were transformed by electroporation of a plasmid carrying the bioluminescence genes. Transposon plasmid pXX3 (constructed with the *lux* operon (*luxCDABE*) and associated chloramphenicol resistance gene) was used (Xu et al. 2010). A stable and virulent bioluminescent strain was selected and used to study colonization of melon plants via leaf, stem, and root inoculations. The variety used in these studies was ‘Athena F1’. A very interesting video of the colonization process via leaf inoculation can be found in the Supplementary Video S1 in Vrisman et al. (2016). A summary of

the colonization process from leaf inoculation is described in Figure 1 (refer to Vrisman et al. (2016) for results on stem and root inoculations). Movement of bacteria into the pedicel of inoculated leaves was detected as early as 1 Day Post Inoculation (DPI). *Erwinia tracheiphila* reached the main stem of melon plants 2 DPI. Based on bioluminescence, bacterial colonization of the main stem starts 2 DPI, culminating in symptom development between 3 to 4 DPI (Figure 1). Colonization with this pathogen in young plants is an irreversible process.

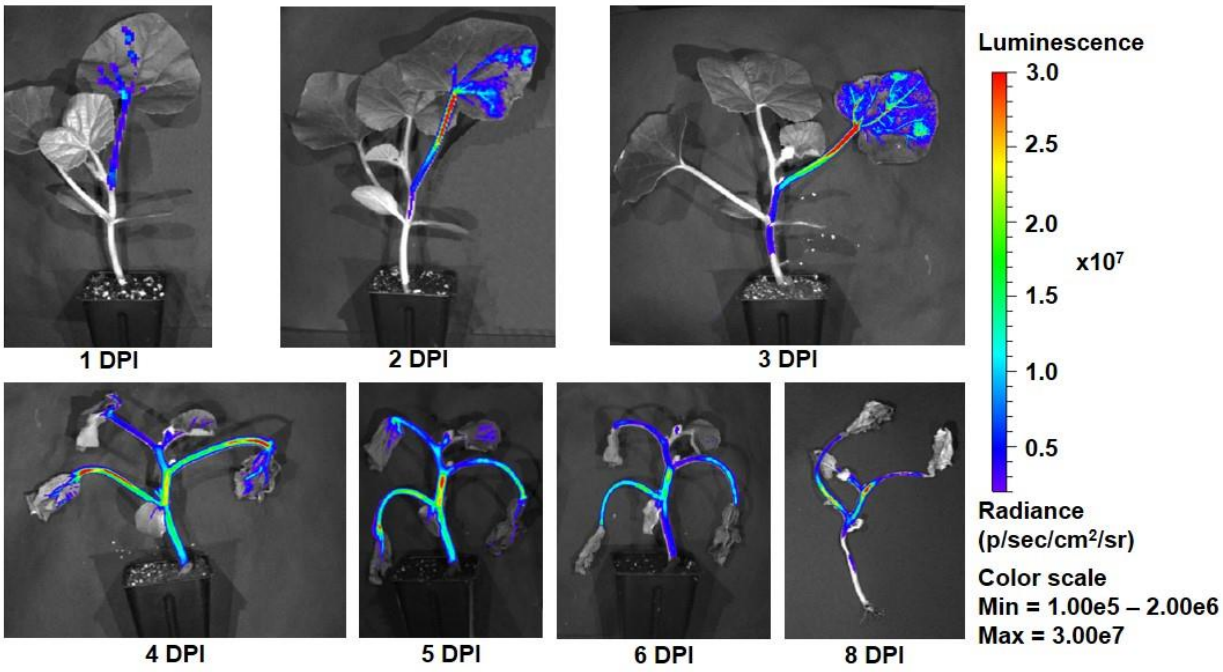

Figure 1. Melon 'Athena F1' plants colonized with a bioluminescent *Erwinia tracheiphila* strain. Details of the inoculation process can be found in Vrisman et al. (2016). The rainbow scale on the right represents light intensity in photons/s. The more light displayed, the more bacterial cells present. DPI = days post inoculation.

With the use of bioluminescence imaging, Vrisman et al. (2016) reported for the first time root colonization resulting from leaf infection (Figure 2). Root colonization after root inoculation was first reported 100 years ago and it has been largely ignored since then. Wounded roots yielded

62.5% colonization success while only 12.5% success was reported with non-wounded roots (Rand and Enlows 1916). Cucumber beetles feed on the roots during their larval stage, although it is not known if larvae can vector this pathogen via roots or if the bacterium can be transmitted from adults to eggs and subsequently to larvae (Vrisman et al. 2016).

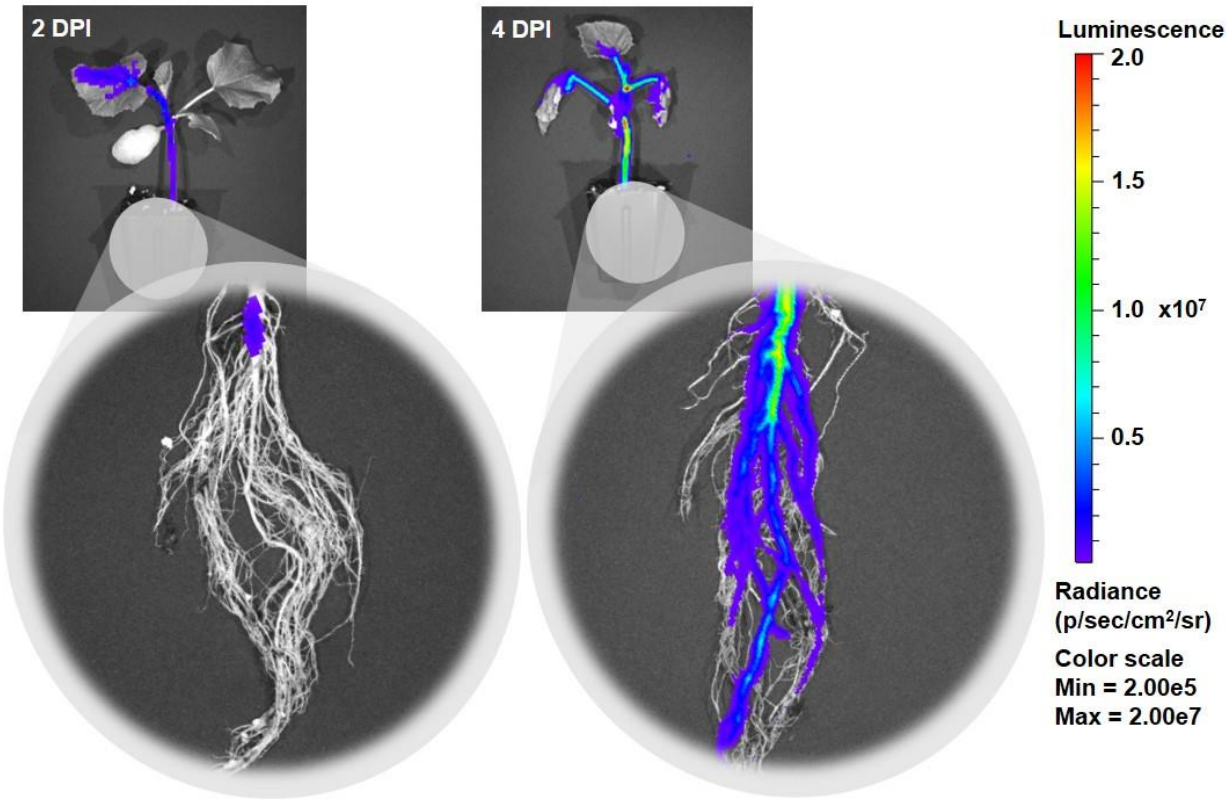

Figure 2. Melon 'Athena F1' plants with roots colonized by a bioluminescent *Erwinia tracheiphila* strain. Details of the inoculation process can be found in Vrisman et al. (2016). The rainbow scale on the right represents light intensity in photons/s. The more light displayed, the more bacterial cells present. DPI = days post inoculation.

Erwinia tracheiphila is known to survive in the digestive tract of its cucumber beetle vectors (de Mackiewicz et al. 1998). Cucumber beetles diapause as adults in the soil during the winter. Beetles emerge in the spring when they start to feed on cucurbits and, potentially, transmit

the pathogen (Shapiro 2012). Bacteria were previously found in the foregut and hindgut of cucumber beetles (Garcia-Salazar et al. 2000). Recent research has shown that this pathogen can survive at least 28 days inside beetles after acquisition with a population of 10^5 colony forming units (CFU) per beetle (Shapiro et al. 2014).

This pathogen has been considered a single species; however, notable differences in the rep-PCR fingerprint profiles, especially between *Cucumis* and *Cucurbita* genera, have been reported recently (Rojas et al. 2013). Given the preference of *E. tracheiphila* strains for hosts in the genus from which they were isolated (Smith, 1911, Rojas et al. 2013, Nazareno and Dumenyo 2015, Vrisman et al. 2016), it has been proposed that this pathogen is undergoing a rapid evolution leading to host specialization (Shapiro 2012) and occurrence of this disease in areas and crops previously unknown for this pathosystem (Sanogo, Etarock, and Clary 2011).

Studies have relied solely on symptom expression to evaluate the host preference among strains of *E. tracheiphila* (Rojas et al. 2013, Nazareno and Dumenyo 2015). Vrisman et al. (2016) were the first to quantify the population of a *Cucumis*-strain inoculated in melon, cucumber, squash, and pumpkin. Based on the bioluminescence signal, the authors detected movement of a *Cucumis*-derived strain into the main stem of squash and pumpkin plants as early as 4 DPI. The signal was still detected at 6 DPI, but disappeared at 8 DPI. Plants did not wilt throughout the length of the experiment, which was 35 days (see Supplementary Fig. S2 in Vrisman et al. (2016) for more details).

Melon and cucumber harbored more than 10^8 CFU/g of stem tissue when bacterial populations were quantified 6 DPI. When non-preferred squash and pumpkin hosts were inoculated with this bioluminescent strain, bacterial populations were approximately 10^4 CFU/g of squash stem tissue and 10^1 CFU/g of pumpkin tissue. Bacterial populations found in squash

stems represent 0.0001% of populations detected when this strain was inoculated in the preferred hosts melon and cucumber. In this study, a strain known to cause disease in squash and pumpkin (BHKY) was used as a control. When this strain was inoculated in squash and pumpkin, its population inside the stem exceeded 10^8 CFU/g of tissue. Detailed results of these finding can be found in Vrisman et al. (2016).

Interestingly, the *Cucumis* strain of *E. tracheiphila* non-productively colonized the stem of squash and pumpkin, and the roots of squash, even 35 DPI in this study; however, no wilting symptoms were observed. Similarly, no wilting symptoms were detected in cross-inoculations performed by Rojas et al. (2013), and Nazareno and Dumenyo (2015). Root colonization in preferred and non-preferred hosts could play an important role in the dissemination of this pathogen and should be further investigated. Larvae of cucumber beetles live in the soil and feed on the roots. It is not known if beetle larvae can acquire this pathogen while feeding on the roots, or if roots can function as an overwintering site for this important disease of cucurbits (Vrisman et al. 2016).

Erwinia tracheiphila is an understudied pathogen of great economic importance. Recent findings on host preference, non-productive colonization of non-preferred hosts, and root colonization helped to better understand the biology of this pathosystem, but also generated more questions that, if addressed, will better elucidate the epidemiology of this rapidly evolving pathogen.

References:

Agrios, G. N. 2005. Plant diseases caused by prokaryotes: bacteria and mollicutes. In Plant Pathology, San Diego: Elsevier Academic Press, p. 639–641.

Azegami, K., Tsukamoto, T., Matsuura, T., Ohara, T., Inoue, Y., Mizuno, A., Yoshida, K., Bessho, H., Kimura, S., Goto, M. 2004. Invasion and colonization of mature apple fruit by *Erwinia amylovora* tagged with bioluminescence genes. J. Gen. Plant Pathol. 70:336–341.

Bogs, J., Bruchmüller, I., Erbar, C., and Geider, K. 1998. Colonization of host plants by the fire blight pathogen *Erwinia amylovora* marked with genes for bioluminescence and fluorescence. Phytopathology. 88:416–421.

Brust, G. E. 1997a. Differential susceptibility of pumpkins to bacterial wilt related to plant growth stage and cultivar. Crop Prot. 16:411–414.

Brust, G. E. 1997b. Interaction of *Erwinia tracheiphila* and muskmelon plants. Environ. Entomol. 26:849–854.

Cruz, A. P. Z., Ferreira, V., Pianzzola, M. J., Siri, M. I., Coll, N. S., and Valls, M. 2013. A novel, sensitive method to evaluate potato germplasm for bacterial wilt resistance using a luminescent *Ralstonia solanacearum* reporter strain. Mol. Plant. Microbe Interact. 27:277–285.

de Mackiewicz, D., Gildow, F. E., Blua, M., Fleischer, S. J., and Lukezic, F. L. 1998. Herbaceous weeds are not ecologically important reservoirs of *Erwinia tracheiphila*. Plant Dis. 82:521–529.

Garcia-Salazar, C., Gildow, F. E., Fleischer, S. J., Cox-Foster, D., and Lukezic, F. l. 2000. Alimentary canal of adult *Acalymma vittata* (Coleoptera: Chrysomelidae): morphology and potential role in survival of *Erwinia tracheiphila*. Can. Entomol. 132:1–13.

Hasegawa, M., Azegami, K., Yoshida, H., and Otani, H. 2003. Behavior of *Erwinia ananas* transformed with bioluminescence genes on rice plants. J. Gen. Plant Pathol. 69:267–270.

Kassem, I. I., Splitter, G. A., Miller, S. A., and Rajashekara, G. 2014. Let there be light! Bioluminescent imaging to study bacterial pathogenesis in live animals and plants. In Advances in Biochemical Engineering/Biotechnology, Springer Berlin Heidelberg, p. 1–27.

Koike, S. T., Gladders, P., and Paulus, A. O. 2007. Diseases of vegetable crops: Cucurbitaceae. In Vegetable Diseases: A Color Handbook, Academic Press, p. 222–223.

Liu, Q., Rojas, E. S., Batzer, J. C., and Gleason, M. L. 2013. Impact of plant age on development of bacterial wilt on muskmelon. Phytopathology. 103(Suppl. 2):S2.83.

Meighen, E. A. 1993. Bacterial bioluminescence: organization, regulation, and applications of the lux genes. The FASEB Journal - Reviews – Department of Biochemistry, McGill University, Montreal, Quebec, Canada.

NASS, (National Agricultural Statistics Service). 2015. USDA Vegetables 2014 Summary (January 2015).

Nazareno, E. S., Dumenyo, C. K. 2015. Modified inoculation and disease assessment methods reveal host specificity in *Erwinia tracheiphila*-Cucurbitaceae interactions. Microbial Pathogenesis 89: 184 – 187.

Rand, F. V., and Enlows, E. M. A. 1916. Transmission and control of bacterial wilt of cucurbits. J. Agric. Res. 6:417–434.

Rojas, E. S., Batzer, J. C., Beattie, G. A., Fleischer, S. J., Shapiro, L. R., Williams, M. A., Bessin, R., Bruton, B. D., Boucher, T. J., Jesse, L. C. H., Gleason, M. L. 2015. Bacterial wilt of cucurbits: resurrecting a classic pathosystem. Plant Dis. 99:564–574.

Rojas, E. S., Dixon, P. M., Batzer, J. C., and Gleason, M. L. 2013. Genetic and virulence variability among *Erwinia tracheiphila* strains recovered from different cucurbit hosts. Phytopathology. 103:900–905.

Sanogo, S., Etarock, B. F., and Clary, M. 2011. First report of bacterial wilt caused by *Erwinia tracheiphila* on pumpkin and watermelon in New Mexico. Plant Dis. 95:1583–1583.

Sebastian, P., Schaefer, H., Telford, I. R. H., Renner, S. S. 2010. Cucumber (*Cucumis sativus*) and melon (*C. melo*) have numerous wild relatives in Asia and Australia, and the sister species of melon is from Australia. PNAS 107: 14269 – 14273.

Shapiro, L. 2012. A to ZYMV guide to *Erwinia tracheiphila* infection: An ecological and molecular study. Published doctoral dissertation. The Pennsylvania State University, University Park, PA.

Shapiro, L., De Moraes, C. M., Stephenson, A. G., Mescher, M. C. 2012. Pathogen effects on vegetative and floral odours mediate vector attraction and host exposure in a complex pathosystem. Ecology Letters 15:1430-1438.

Shapiro, L. R., Seidl-Adams, I., De Moraes, C. M., Stephenson, A. G., and Mescher, M. C. 2014. Dynamics of short- and long-term association between a bacterial plant pathogen and its arthropod vector. Sci. Rep. 4:4155.

Smith, E. F. 1911. Wilt of cucurbits. In Bacteria in relation to plant diseases. Carnegie Institution Washington Publications, Washington, DC, p. 209–299.

Stewart, G. S. A. B.; Williams, P. 1992. Lux genes and the applications of bacterial bioluminescence. Journal of General Microbiology, vol. 138, 1289 – 1300.

Vrisman, C. M., Deblais, L., Rajashekara, G., Miller, S. A. 2016. Differential colonization dynamics of cucurbit hosts by *Erwinia tracheiphila*. Phytopathology (http://dx.doi.org/10.1094/PHYTO-11-15-0289-R).

Xu, X., Miller, S. A., Baysal-Gurel, F., Gartemann, K.-H., Eichenlaub, R., and Rajashekara, G. 2010. Bioluminescence imaging of *Clavibacter michiganensis* subsp. *michiganensis* infection of tomato seeds and plants. Appl. Environ. Microbiol. 76:3978–3988.

Xu, X., Rajashekara, G., Paul, P. A., and Miller, S. A. 2011. Colonization of tomato seedlings by bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* under different humidity regimes. Phytopathology. 102:177–184.

Yao, C., Zehnder, G., Bauske, E., and Kloepper, J. 1996. Relationship between cucumber beetle (Coleoptera: Chrysomelidae) density and incidence of bacterial wilt of cucurbits. J. Econ. Entomol. 89:510–514.